Current Biology, Vol. 13, 758–762, April 29, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/S0960-9822(03)00286-0

A Novel C-Terminal Domain of *Drosophila* PERIOD Inhibits dCLOCK:CYCLE-Mediated Transcription

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Summary

The essence of the *Drosophila* circadian clock involves an autoregulatory feedback loop in which PERIOD (PER) and TIMELESS (TIM) inhibit their own transcription by association with the transcriptional activators dCLOCK (dCLK) and CYCLE (CYC) [1]. Because PER, dCLK, and CYC each contain a PAS domain [1], it has been assumed that these interaction domains are important for negative feedback. However, a critical role for PAS-PAS interactions in *Drosophila* clock function has not been shown. Nuclear transport of PER is also believed to be an essential regulatory step for negative feedback [1–3], but this has not been directly tested, and the relevant nuclear localization sequence (NLS) has not been functionally mapped. We evaluated these critical aspects of PER-mediated transcriptional inhibition in *Drosophila* Schneider 2 (S2) cells. We mapped the dCLK:CYC inhibition domain (CCID) of PER and discovered that it lies in the C terminus, downstream of the PAS domain. Using deletion mutants and site-directed mutagenesis, we identified a novel NLS in the CCID of PER that is a potent regulator of PER's nuclear transport in S2 cells. We further found that nuclear transport, primarily through this novel NLS, is essential for the inhibitory activity of PER. The data indicate that nuclear PER inhibits dCLK:CYC-mediated transcription through a novel domain that additionally contains a potent NLS.

Results and Discussion

PER Inhibits dCLK:CYC Independent of TIM

The PER:TIM complex is believed to inhibit dCLK:CYC through a direct physical interaction with the transcription factors, as suggested by in vitro gel shift assays [4]. PER, dCLK, and CYC each possess a PAS domain [1], consisting of two PAS motifs of approximately 50 amino acids each (PAS-A and PAS-B), a linker region between them, and a region downstream of PAS-B called PAC, which in PER acts as a cytoplasmic localization domain (CLD) [5]. PAS domains have been implicated in protein-protein interactions [6], and indeed the PAS domain of PER has been shown to bind TIM [7, 5]. Thus, it seemed likely that PER:TIM inhibits dCLK:CYC transcriptional activity through PAS domain interactions.

Drosophila Schneider 2 (S2) cells are a useful system for testing this hypothesis because dCLK:CYC transcriptional activation via E boxes (using transiently transfected *dClk* and endogenously expressed *cyc*) and PER:TIM inhibition of this activation can be reconstituted in these cells [8]. Previous S2 cell culture studies utilized the coexpression of PER and TIM to inhibit dCLK:CYC-mediated transcription because PER and TIM appeared to be codependent for nuclear localization [5] and robust inhibition of dCLK:CYC [8]. However, recent in vivo data indicate that PER in the absence of TIM inhibits dCLK:CYC-mediated transcription [9] and that TIM may not be essential for PER nuclear transport [3]. Accordingly, we reexamined the inhibitory activity of PER in an S2 cell transcriptional assay, alone, and with TIM at different plasmid concentrations (Figure 1).

Consistent with the idea that PER alone is sufficient for inhibition of dCLK:CYC-mediated transcription, we found that in S2 cells, PER without TIM inhibited dCLK:CYC to more than 90% at high doses (100 ng and 600 ng; Figure 1). TIM without PER only modestly inhibited dCLK:CYC, attaining maximal inhibition of $<$40% (Figure 1). Consistent with previous work [8], TIM enhanced PER's activity at low levels of PER (10 ng plasmid, the same concentration used earlier by others [8]), possibly by facilitating PER nuclear transport and/or stability [1]. However, higher doses of PER may exceed the capacity of cytoplasmic localization and/or degradative mechanisms, allowing accumulation of enough nuclear PER to inhibit dCLK:CYC robustly, even in the absence of TIM. Thus by using high plasmid doses (200 ng) in subsequent experiments, we could study PER's inhibitory activity without dependence on TIM binding sites for enhancement of PER nuclear transport and/or stability. Furthermore, because V5-tagged PER was found to be as effective as untagged PER for transcriptional repression (Figure 1), we used V5-tagged constructs for the rest of our studies, facilitating the determination of relative protein abundance and subcellular location.

A Novel C-Terminal Domain in PER Inhibits dCLK:CYC-Mediated Transcription

To identify the region of PER responsible for inhibiting dCLK:CYC-mediated transcription, we tested deletion mutants (at a plasmid dose of 200 ng) in the S2 cell transcription assay (Figure 2). All of the constructs studied yielded comparable levels of protein expression (data not shown). To our surprise, we found that fragments containing the PAS domain did not inhibit dCLK:CYC whereas a PER fragment downstream of the PAS domain (PER 513–1224) possessed robust inhibitory activity. Using additional deletion mutants, we mapped the dCLK:CYC inhibition domain (CCID), defined as the minimal region producing over 50% inhibition under the conditions tested, to amino acid residues

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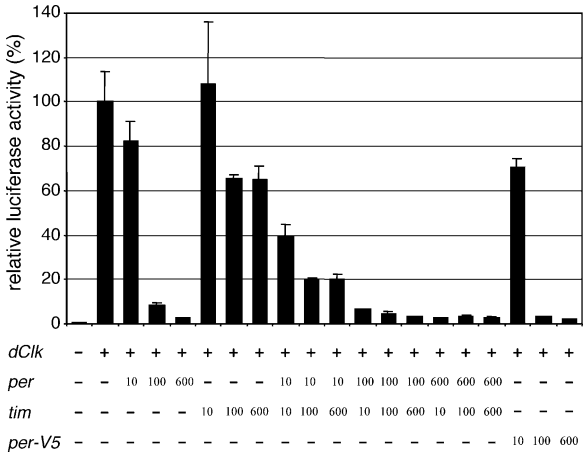

Figure 1. PER Alone Inhibits dCLK:CYC-Mediated Transcription in S2 Cells

The presence (+) or absence (−) of expression plasmids (1 ng *dClk*; 10 ng, 100 ng, or 600 ng of *per*, *tim*, or V5 epitope-tagged *per* (*per-V5*)) is depicted. CYC is expressed endogenously in S2 cells [8]. The luciferase activity (expression driven by a minimal hsp70 promoter and four *per* E boxes) relative to the β-galactosidase activity (expression driven by an actin promoter) was computed and normalized such that the mean value in the presence of dCLK alone was 100%. Each value is mean ± SEM of three replicates. The results shown are representative of three independent experiments.

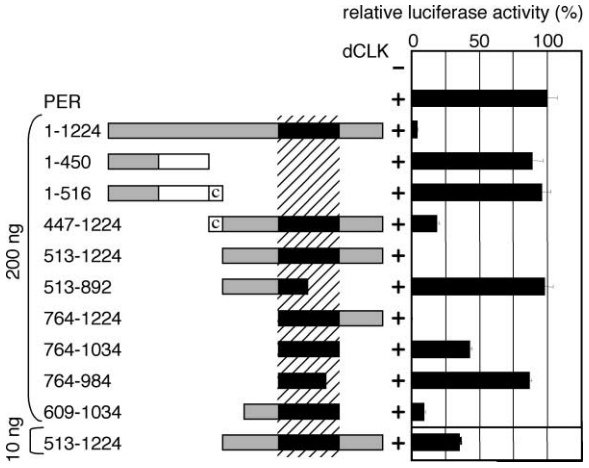

Figure 2. Mapping of the dCLK:CYC Inhibition Domain of PER

S2 cells were transfected with 1 ng *dClk* and 200 ng or 10 ng of *per* full-length or deletion mutant constructs. The luciferase activity relative to the β-galactosidase activity was computed and normalized such that the mean value in the presence of dCLK alone was 100%. Each value is mean ± SEM of three replicates. The numbers associated with each mutant (illustrated schematically as bars) represent the amino acids of full-length PER (1224 aa) contained in each mutant protein. PAS domain, white box; CLD, white box labeled "c"; CCID, black box in hatched region. The results shown are representative of three independent experiments.

764–1034 of PER (Figure 2; numbering according to the 1224 aa form of PER: splice variant A of the Canton-S strain, GenBank accession number P07663). Fragments PER 764–1224 and PER 609–1034 had higher inhibitory activity than PER 764–1034 (Figure 2), suggesting that flanking regions of the protein, although not essential, may enhance the inhibitory activity or the structural stability of the CCID. To ensure that the inhibition we observed was not artificially generated by overexpression of our PER constructs, we retested PER 513–1224 at a lower dose (10 ng) and found that it still achieved potent ≥70% inhibition (Figure 2).

A Potent NLS Resides in the CCID of PER

We next examined both the CLD and functional nuclear localization sequences (NLSs) within PER because regulated nuclear entry is thought to be an important component of the transcriptional feedback mechanism [1]. As previously mentioned, PER has a CLD at the C-terminal end of its PAS domain (residues 452–512) [5]. Sequence analysis predicts that PER possesses at least two NLSs of the classical basic monopartite variety: one near the N terminus (aa 73–77; "NLS73-7") [10] and one in the C-terminal half of the protein (aa 788–791; "NLS788-91") [11]. However, neither of the predicted NLSs has been verified to be a functional NLS.

We used immunocytochemistry to examine the subcellular localization of various V5-tagged PER deletion mutants expressed in S2 cells. For each cell examined, the V5-tagged protein was classified as having one of three staining patterns: nuclear (N), cytoplasmic (C), or both nuclear and cytoplasmic (B). Examples of each staining pattern are shown in Figure 3B. The proportion of cells in each category (N, C, or B) relative to the total number of cells examined for that construct was calculated as a percentage. Full-length PER was primarily cytoplasmic (in 69% of cells; Figure 3A; see Table S1 in the Supplemental Data available with this article online), consistent with observations by others [5]. Moreover, we confirmed that a CLD was located between amino acids 450 and 513 since the fragment PER 1–450 had a greater degree of nuclear localization than did PER 1–516 (83% of cells stained in both cytoplasm and nucleus versus 59% cyptoplasm only, respectively), and PER 513–1224 had a greater degree of nuclear localization than did PER 447–1224 (91% nucleus only versus 57% both cytoplasm and nucleus, respectively; Figure 3A and Table S1).

Because the presence of a CLD in PER could obscure the study of its NLSs and because there appear to be multiple NLSs, we analyzed NLS function in separate PER deletion mutants, one that is upstream and one that is downstream of the CLD. In PER 1–450, we mutated the basic amino acids in NLS73-7 to alanine ("1–450 M73-7"), while in PER 513–1224, we mutated the basic amino acids in NLS788-91 to alanine ("513–1224 M788-91"). To our surprise, these mutations had only small effects on subcellular localization (Figure 3A; Table S1).

We therefore assessed the localization of additional deletion mutants. We found that a functional NLS mapped to a C-terminal region between amino acids 813 and 840 (Figure 3A). Basic amino acids are characteristic of classic NLSs recognized by importin-α/β [12], and, in this region, we found basic amino acids in two clusters, 822–823 and 835–837, with a spacing (11 aa) consistent with a classical bipartite NLS [12] (Figure 3A). When the basic amino acids in either of these clusters were mutated to alanine in PER 513–1224 (generating

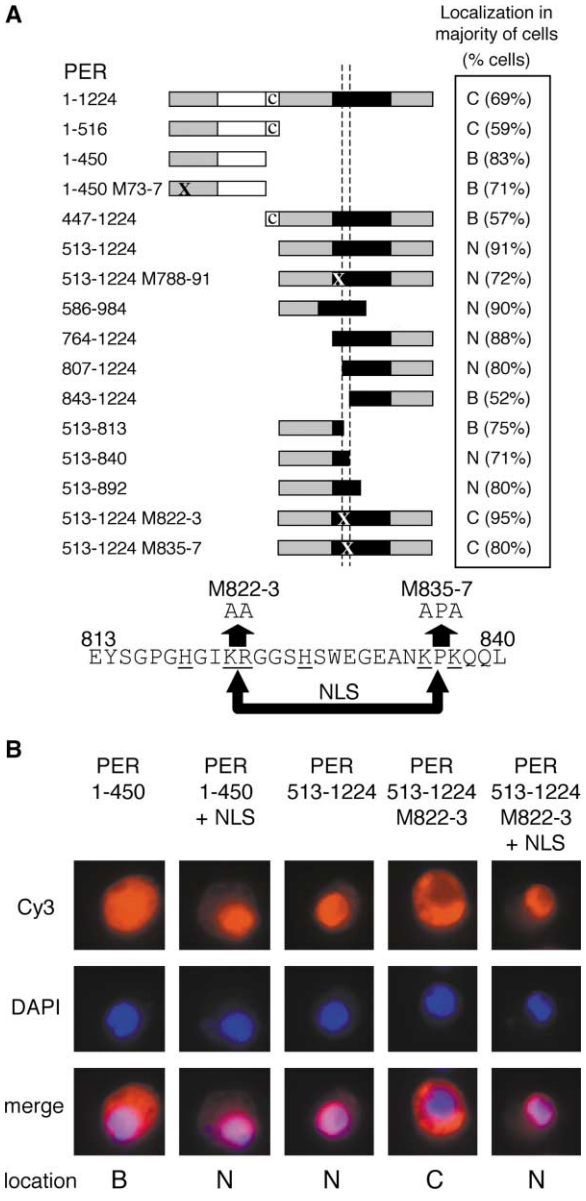

Figure 3. Mapping a Novel Nuclear Localization Sequence

V5-tagged *per* constructs (ca. 300 ng) were transfected into S2 cells and the cellular location of their protein products was assayed by immunocytochemistry using a monoclonal anti-V5 primary antibody and a Cy3-conjugated secondary antibody. The cells were also DAPI stained to visualize the nuclei.

(A) For each cell examined, the V5-tagged protein was classified as having one of three staining patterns: nuclear (N), cytoplasmic (C), or both nuclear and cytoplasmic (B). For each construct, the proportion of cells in each category (N, C, or B) relative to the total number of cells examined was calculated as a percentage; the immunostaining pattern (N, C, or B) of >50% of cells examined is shown in the right-hand column, and the percentage of cells with that staining pattern is shown in parentheses (complete data are given in Table S1). The vertical dashed lines indicate the NLS-containing region (aa 813–840) determined using deletion mutants. The sequence of this region is shown below the constructs. The basic amino acids (lysine, K; arginine, R; histidine, H) are underlined, mutations to alanine are indicated above the sequence, and functional components of the bipartite NLSs, as shown by mutagenesis, are indicated below the sequence. PAS domain, white box; CLD, white box labeled "c"; CCID, black box; mutagenized putative NLS, "X."

(B) Representative subcellular staining patterns of PER constructs. Cy3 (red) and DAPI (blue) signals were imaged separately (first and second rows of images, respectively) using wavelength-selective filters on a fluorescence microscope and digitally merged (overlap is magenta; bottom row of images) using the Metamorph software package (Universal Imaging Corp.). "+ NLS" denotes constructs tagged with two SV40 large T-antigen NLSs.

"PER 513–1224 M822-3" and "PER 513–1224 M835-7"), the protein became predominantly cytoplasmic (Figures 3A and 3B; Table S1). Thus, PER has a functional bipartite NLS in its C-terminal region within the CCID. It is important to note that the N-terminal fragments of PER (1–450 and its mutated counterpart, 1–450 M73-7) were detectable in the nuclei of S2 cells (71%–83% stained in both cytoplasm and nucleus; Figure 3A; Table S1), suggesting the presence of secondary NLSs or N-terminal binding sites with other proteins that could facilitate nuclear transport.

The combined cytoplasmic *and* nuclear presence of a PAS-containing PER (1–450) fragment (Figures 3A and 3B) suggests that a lack of nuclear transport cannot explain the failure of the PAS domain to inhibit dCLK:CYC-mediated transcription. We confirmed that the PER PAS domain does not inhibit transcription by tagging PAS-containing N-terminal fragments of PER (1–516 and 1–450) with a strong heterologous NLS (two tandem repeats of the SV40 large T-antigen NLS, DKKKRKV, which is a basic monopartite NLS). The NLS-tagged PER 1–516 (which includes the PAS and CLD domains) was found to be in both nucleus and cytoplasm of 60% of transfected cells, while the NLS-tagged PER 1–450 (PAS domain without the CLD) was robustly nuclear (83% of cells; Figures 3B and 4; Table S1). When tested in a transcription assay, these new constructs were still unable to inhibit dCLK:CYC (Figure 4), indicating that the PAS domain does not possess inhibitory activity.

Nuclear Presence of PER Is Essential for Inhibition of dCLK:CYC Transcription

Although in vivo studies have correlated PER nuclear entry with the inhibition of dCLK:CYC-mediated transcription [2, 3], it has not been shown whether PER *must* be nuclear to mediate transcriptional inhibition. In fact, it has been suggested that cytoplasmic PER:TIM could inhibit dCLK:CYC by sequestering one or both of the transcription factors in the cytoplasm, a scenario demonstrated for other transcription factors [4]. The ability of PER alone to inhibit dCLK:CYC in S2 cells, despite its primarily cytoplasmic localization (Figures 1 and 3), is consistent with the cytoplasmic sequestering idea.

To examine whether nuclear entry is indeed necessary for PER's inhibitory activity, we further evaluated the NLS mutant constructs of PER 513–1224 (PER 513–1224 M822-3 and M835-7). We found that the NLS mutant proteins, which were cytoplasmic in ≥80% of transfected cells (Figures 3 and 4; Table S1), failed to inhibit dCLK:CYC-mediated transcription, suggesting that the nuclear presence of the PER CCID is required for transcriptional inhibition (Figure 4). However, because the NLS is internal to the CCID, it remained possible that

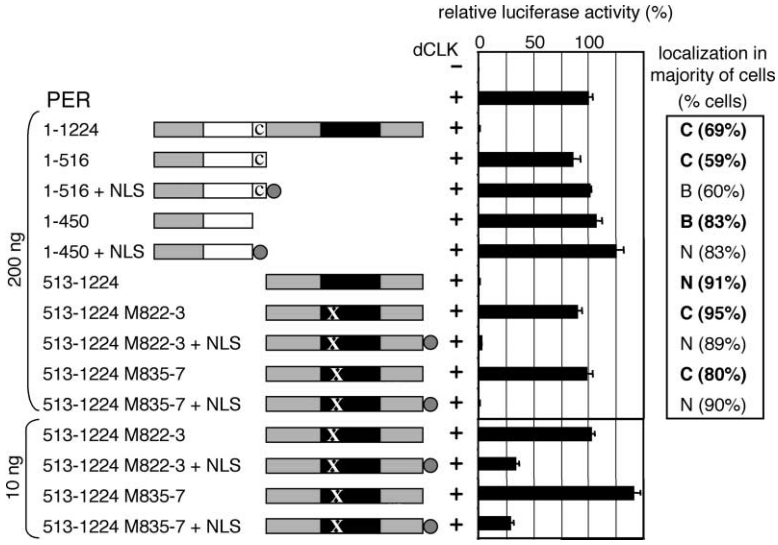

Figure 4. Nuclear Presence of the CCID Is Required for PER Inhibition of dCLK:CYC-Mediated Transcription

V5-tagged *per* constructs (left column) were assayed for inhibition of dCLK:CYC-mediated transcription (middle column) and cellular location via immunocytochemistry (right column). Transcription assays used 1 ng *dClk* and 200 ng or 10 ng of the *per* constructs. The luciferase activity relative to the β-galactosidase activity was computed and normalized such that the mean value in the presence of dCLK alone was 100%. Each value is mean ± SEM of three replicates. For each cell examined by immunocytochemistry, the V5-tagged protein was classified as having one of three staining patterns: nuclear (N), cytoplasmic (C), or both nuclear and cytoplasmic (B). For each construct, the proportion of cells in each category (N, C, or B) relative to the total number of cells examined was calculated as a percentage; the immunostaining pattern (N, C, or B) of >50% of cells examined is shown in the right column, and the percentage of cells with that staining pattern is shown in parentheses (complete data are given in Table S1). The immunocytochemical data in bold have been duplicated from Figure 3. PAS domain, white box; CLD, white box labeled "c"; CCID, black box; mutagenized NLS, "X"; SV40 large T-antigen NLS (DKKKRKV) × 2, circles. The results shown are representative of three independent experiments.

the NLS mutations disrupted the structure of the CCID in addition to preventing nuclear transport. We thus tried to rescue inhibitory activity by tagging the C terminus of the NLS mutant constructs with SV40 large T-antigen NLSs. Not only were these new constructs robustly nuclear, but they now inhibited dCLK:CYC as effectively as the wild-type PER 513–1224, indicating that the NLS mutations did not disrupt CCID structure (Figures 3B and 4; Table S1). We obtained similar results with low doses (10 ng) of the NLS-tagged and untagged PER 513–1224 mutant and wild-type plasmids (Figure 4). The dependence of PER's inhibitory activity on nuclear presence explains why the robustly nuclear PER 513–1224 is more effective at inhibiting dCLK:CYC than comparable doses of primarily cytoplasmic, full-length PER (Figure 2). We conclude that the nuclear presence of PER (or a PER fragment containing the CCID) is essential for its inhibitory action on dCLK:CYC-mediated transcription.

Conclusions

We found that a key step in the *Drosophila* circadian negative feedback loop, PER inhibition of dCLK:CYC transcription, is not mediated by the PAS domain of PER. Instead, we discovered that the previously uncharted C terminus of PER contains a novel domain (CCID; aa 764–1034) responsible for transcriptional inhibitory activity (Figures 2 and 5). The functional importance of this C-terminal region of PER is corroborated by an earlier in vivo experiment, in which a *per* transgene extending only up to amino acid 876 failed to rescue behavioral rhythms in *per* null mutant (*per*^{01}) flies [13]. This truncated PER would still possess binding sites for TIM [5, 7], DOUBLE-TIME [14], and CRYPTOCHROME [15], but, as our experiments reveal, its CCID would be disrupted (Figure 5). We also found that the monopartite NLSs previously predicted by sequence analysis are relatively weak in regulating PER localization. Instead, a novel, bipartite NLS in the CCID is the dominant NLS in S2 cells (Figure 3A). However, there may be several NLSs that contribute to PER nuclear transport in vivo since transgenic fly experiments suggest that there is a competent NLS in the first 95 amino acids of PER [16], and N-terminal fragments of PER do show some nuclear localization in S2 cells (Figure 3A). We further demonstrated that the nuclear transport of PER is essential for its inhibition of dCLK:CYC-mediated transcription (Figure 4), as suggested by earlier work [2, 3]. These results advance our understanding of PER function and thus our understanding of the *Drosophila* circadian clock mechanism.

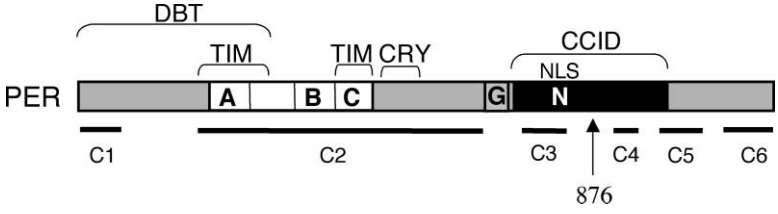

Figure 5. PER Subdivisions and Functional Domains

PAS domain, white box with the PAS-A ("A"), PAS-B ("B"), and CLD "C" indicated; CCID, black box; glycine-threonine repeat, box with the letter "G." Brackets above PER indicate sites of demonstrated interaction with other clock proteins: DBT = DOUBLE-TIME [14], TIM = TIMELESS [5, 7], CRY = dCRYPTOCHROME [15], and CCID = dCLK:CYC inhibitory domain (Figure 2). The arrow indicates the location of amino acid 876; a transgene truncated at this residue fails to rescue behavioral rhythms in *per*^{01} mutant flies [13]. Black lines underneath PER indicate the regions conserved in PER proteins of other insect species: C1 through C6, as defined by Colot and colleagues [17].

Supplemental Data

Supplemental Data including detailed Experimental Procedures and immunocytochemical data (Table S1) are available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

We thank Joel Levine and Sriram Sathyanarayanan for help generating some of the constructs and Aditi Chavda and Kurtis Gray for technical assistance. D.C.C. was supported by a Howard Hughes Medical Institute Predoctoral Fellowship in Biological Sciences. This work was funded by NIH grant NS 39303.

Received: February 10, 2003
Revised: February 28, 2003
Accepted: March 3, 2003
Published: April 28, 2003

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